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Involvement of thromboxane A_2, leukotrienes and free radicals in puromycin nephrosis in rats

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Involvement of thromboxane A_2, leukotrienes and free radicals in puromycin nephrosis in rats. Thromboxane A_2 (TXA_2), leukotrienes (LTs) and free radicals are considered to be possible mediators in the induction of glomerular injury and proteinuria. In this study, we examined the involvement of these three mediators and the protective effect of simultaneous inhibition of all three in puromycin aminonucleoside (PAN) nephrosis in rats. A single intraperitoneal injection of PAN (100 mg/kg) induced massive proteinuria and enhanced production of TXA_2 and LTs from arachidonic acid in renal cortical slices and renal glomeruli, and increased malondialdehyde levels in plasma, urine and renal cortex. Oral administration of CV-6504(HCl) (3 to 20 mg/kg/day, for 1 to 2 weeks), a novel treble inhibitor of TXA_2 synthetase, 5-lipoxygenase and lipid peroxidation, dose-dependently attenuated PAN-induced proteinuria and the increases in these three mediators. Any single specific inhibitor (CV-4151, a TXA_2 synthetase inhibitor; AA-861, a 5-lipoxygenase inhibitor; or CV-3611, a radical scavenger) or a combination of two inhibitors showed no or only a slight antiproteinuric effect, but the combination of all three inhibitors significantly reduced PAN-induced proteinuria. These results suggest that, these three mediators may be involved in the pathogenesis of PAN nephrosis and that CV-6504(HCl), which can simultaneously inhibit all three, may be a useful therapeutic agent for nephrosis.

Several recent studies have indicated that reactive oxygen metabolites [1–5], thromboxane A_2 (TXA_2) [6, 7] and leukotrienes (LTs) [8–11] may play important roles in the pathophysiology of renal glomerular diseases. A variety of inhibitors of these mediators are known to protect against glomerular injury and proteinuria in various forms of experimental nephropathy.

A single i.p. injection of puromycin aminonucleoside (PAN) in rats causes marked proteinuria and a glomerular morphologic abnormality (epithelial cell injury) that are similar to minimal change nephrotic syndrome in humans [12]. Recently, Diamond, Bonventre and Karnovsky [4] postulated that the glomerular injury associated with PAN treatment is mediated by generation of oxygen free radicals. In addition to oxygen free radicals, the metabolites of arachidonic acid such as TXA_2 and LTs may be involved in the pathogenesis of PAN nephrosis, because oxygen free radicals are known to stimulate the production of TXA_2 and prostaglandin E_2 in cultured rat mesangial cells [13], and because PAN treatment also activates phospholipase which digests membrane phospholipids causing the release of arachidonic acid (AA; a precursor of TXA_2 and LTs) [14]. Enhanced synthesis of TXA_2 and prostaglandins has been reported to influence renal function and hemodynamics [6, 15–20]. However, the nature of renal production of TXA_2 and LTs and renal lipid peroxidation has not yet been examined at the same time in PAN-induced nephrosis.

CV-6504(HCl), 2,3,5-trimethyl-6-(3-pyridylmethyl)-1,4-benzoquinone hydrochloride, has three different pharmacologic actions: it inhibits TXA_2 synthetase and 5-lipoxygenase, and scavenges free radicals [21]. If all three of the above-mentioned mediators are involved in PAN-induced nephrosis, an agent such as CV-6504(HCl), which can simultaneously inhibit all three, should be much more effective in the treatment of the disease than a pure inhibitor of one individual mediator.

Therefore, the aim of the present study is to examine the possible involvement of TXA_2, LTs and lipid peroxidation in PAN-induced nephrosis and the effect of CV-6504(HCl) on the proteinuria.

Methods

Experimental design

Adult male Wistar rats that weighed 120 to 130 g (5 weeks old) and had free access to water and standard rat chow were used in these experiments. Nephrotic syndrome was induced by a single i.p. injection of PAN at a dose of 100 mg/kg body weight [22].

Experiment 1

To investigate the time course of changes in blood, urinary and renal tissue parameters, blood, urine and renal tissue were obtained day 1 ($N = 5$), 3 ($N = 5$), 7 ($N = 5$) and 14 ($N = 7$) after PAN injection. Blood was collected from the abdominal aorta under pentobarbital anesthesia. Plasma samples were subjected to procedures for the measurement of MDA, BUN, total protein, albumin, creatinine and total cholesterol. The kidneys were removed and subjected to a biosynthetic study on renal TXA_2, PGI_2, LTs and MDA as described later.

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Experiment 2

CV-6504(HCl) at doses of 3 (N = 11), 10 (N = 21) and 20 (N = 11) mg/kg was given orally once a day for 14 days. The first dose of CV-6504(HCl) was given one hour prior to the PAN injection. Control rats (N = 10) were given only vehicle (5% gum arabic solution). Urine was collected on days 7 and 14 after PAN injection, and urinary total protein and albumin were measured. On day 14, the effects of CV-6504(HCl) (10 mg/kg) on the changes in various blood and urinary parameters induced by PAN injection, the production of TXA_2 and LTs and the production of MDA in renal cortical slices were analyzed as described later. The kidneys from rats receiving CV-6504(HCl) at 10 mg/kg (p.o.) were also examined histologically day 14 after PAN injection.

Experiment 3

CV-6504(HCl) was administered for 14 days at 10 mg/kg/day (p.o., N = 8) beginning one day after PAN injection. Urine was collected on days 8 and 15 after PAN injection.

Experiment 4

To investigate the in vitro effect of CV-6504(HCl) on the production of TXA_2, LTs and MDA, CV-6504(HCl) was added to the incubation medium of fresh renal cortical slices from control rats day 14 after PAN injection. Further, to estimate the ex vivo effect of a single dose of CV-6504(HCl), CV-4151, AA-861 or CV-3611 on the production of TXA_2, LTs or MDA, each drug was administered to control rats day 14 after PAN injection, and one hour later the kidneys were isolated. The production of TXA_2, LTs or MDA was then analyzed in renal cortical slices or renal glomeruli as described later.

Experiment 5

The effects of the combined administration of CV-4151 (a TXA_2 synthetase inhibitor [23]), AA-861 (a 5-lipoxygenase inhibitor [24]) and CV-3611 (a radical scavenger [25]) on the proteinuria in PAN-induced nephrosis were examined. Nine groups were used: (a) normal rats without PAN injection (N = 7), (b) vehicle (N = 7), (c) CV-4151 10 mg/kg, p.o. (N = 7), (d) CV-3611 50 mg/kg, p.o. (N = 7), (e) AA-861 300 mg/kg, p.o. (N = 7), (f) CV-4151 + CV-3611 (N = 7), (g) AA-861 + CV-3611 (N = 7), (h) AA-861 + CV-4151 (N = 7) and (i) AA-861 + CV-4151 + CV-3611 (N = 7). In groups (b) through (i) the first dose of vehicle or drug was administered one hour prior to PAN injection. At the doses of CV-4151, AA-861 and CV-3611 used in this experiment, significant inhibitory effects on the production of TXA_2, LTs and MDA in renal cortical slices, respectively, were shown in Experiment 4.

Miscellaneous measurements

Urinary protein was measured using a A/G-B test® (Wako, Japan) based on Biuret reaction [26], and urinary and plasma albumin were measured using an Albumin-B test® with bromcresol green [27] (Wako). Plasma cholesterol, BUN and plasma creatinine were measured using a cholesterol E-test® (a modified cholesterol oxidase method by Richmond et al [28, 29]) and a Creatinine test® (Wako) based on the Jaffe reaction [30], respectively. MDA in plasma, urine, renal cortex and the incubation medium of renal cortical slices was measured using a Lipoperoxide test® (Wako) based on thiobarbituric acid (TBA) method [31]. To measure plasma and urinary excretion of 11-dehydro-TXB_2 and LTB_4, urine and plasma were extracted with 4 volumes of ethyl acetate in the tubes including indomethacin (10^{-6} M) for 11-dehydro-TXB_2 [32] and cold methanol for LTB_4 [33]. LTB_4 and 11-dehydro-TXB_2 were measured by RIA using kits (Amersham, Arlington Heights, Illinois, USA).

Preparation and incubation of renal cortical slices or renal glomeruli, and measurement of TXA_2, LTs and MDA

The biosynthesis of renal TXA_2, PGI_2 and LTs and the production of renal MDA were examined as follows: kidneys were perfused with ice-cold Krebs-Henseleit solution (20 ml) to remove blood. The renal cortex of the right kidney was removed quickly and cut into 0.3 mm-thick slices using a tissue chopper (McLwein) or glomeruli were prepared using three consecutive sieves (mesh size 120, 133 and 35 μm) according to the method of Fujiwara et al [34]. Purity of the glomeruli was assessed by light microscopy. Preparations were found to contain more than 95% glomeruli. The slices (about 10 mg) or glomeruli (about 4000) were suspended in 2 ml of Krebs-Henseleit solution (gassed with 95% O_2-5% CO_2). Arachidonic acid (AA) was added at a final concentration of 20 μM. Incubation with or without AA was carried out at 37°C for 30 minutes under continuous agitation (80 cycles/min) and was terminated by centrifugation at 1500 × g for five minutes. The supernatant (0.3 ml) was extracted with 1.2 ml cold methanol to measure LTs, and an aliquot of the supernatant was immediately frozen to measure TXB_2 (the stable metabolite of TXA_2) and 6-keto-$PGF_{1\alpha}$ (the stable metabolite of PGI_2). LTs were measured by radioimmunoassay (RIA) using kits (Amersham) sensitive to LTC_4, LTD_4 and LTE_4. TXB_2 and 6-keto-$PGF_{1\alpha}$ were also measured by RIA using TXB_2 or 6-keto-$PGF_{1\alpha}$ antiserum as described before [23, 35]. Cross reactivity of TXB_2 antiserum with other prostaglandins was less than 0.1% for PGE_1 and PGE_2, 0.6% for $PGF_{2\alpha}$, 0.1% for 6-keto-$PGF_{1\alpha}$ and 1.1% for PGD_2. That of 6-keto-$PGF_{1\alpha}$ antiserum was less than 0.1% for PGA_2, PGD_2 and TXB_2, 1.8% for PGE_1, 1.3% for PGE_2, 4.5% for $PGF_{2\alpha}$, 0.4% for 6, 15-diketo-$PGF_{1\alpha}$ and 0.3% for 6, 15-diketo-13, 14-dihydro-$PGF_{1\alpha}$.

To assay the production of MDA, the residual slices (40 mg) were incubated in 0.5 ml of phosphate-buffered saline at 37°C for 45 minutes, and then the level of MDA in the incubation medium was measured using a Lipoperoxide test as described above. To measure renal content of MDA, the left kidneys were homogenized in 9 volume of phosphate buffered saline, and the level of MDA in the homogenates was measured as described above. The amount of protein in the slices or homogenates was determined by the method of Lowry et al using BSA as the standard [36].

Histological examination

For light microscopic examination, the kidneys were fixed in 10% buffered formalin and embedded in paraffin. Sections 4 μm thick were stained with hematoxylin-eosin and periodic acid Schiff (PAS) and were then examined under a Nikon Microphot light microscope. A portion of the renal tissue was rapidly removed and fixed in ice-cold 3% glutaraldehyde solution. The

Table 1. Time course of the changes in the concentration of BUN, plasma creatinine, plasma albumin and plasma cholesterol after PAN injection in Experiment 1

Days after PAN injection		1	3	7	14
Number of animals		5	5	5	7
BUN	Normal	15.4 ± 1.20	14.8 ± 0.8	15.3 ± 0.9	16.0 ± 0.8
mg/dl	PAN	21.8 ± 0.99[a]	21.9 ± 0.9[a]	94.2 ± 5.8[c]	21.5 ± 1.0[a]
P_{Cr}	Normal	0.400 ± 0.006	0.405 ± 0.002	0.395 ± 0.011	0.406 ± 0.003
mg/dl	PAN	0.410 ± 0.002	0.402 ± 0.001	0.442 ± 0.002[b]	0.414 ± 0.009
P_{alb}	Normal	3.37 ± 0.15	3.19 ± 0.16	3.43 ± 0.07	3.59 ± 0.05
g/dl	PAN	3.16 ± 0.09	3.42 ± 0.09	2.00 ± 0.02[b]	3.19 ± 0.12[a]
P_{chol}	Normal	60.9 ± 2.5	63.4 ± 3.8	54.6 ± 3.8	65.9 ± 4.1
mg/dl	PAN	80.3 ± 8.5	88.4 ± 4.0[a]	330.4 ± 15.8[c]	166.3 ± 15.3[b]

Abbreviations are: PAN, puromycin aminonucleoside, 100 mg/kg, i.p.; P_{Cr}, plasma creatinine concentration; P_{alb}, plasma albumin concentration; P_{chol}, plasma cholesterol concentration.
[a] $P < 0.05$, [b] $P < 0.01$ and [c] $P < 0.001$ vs. normal rat (Student's *t*-test)

specimens were post-fixed in 1% osmic acid, dehydrated through a graded ethanol series, and then embedded in epoxy resin. Ultrathin sections were cut and stained with uranyl acetate and lead citrate, and examined under a JEM 1200EX (Tokyo, Japan) electron microscope. The criteria scoring was as follows.

PAS positive droplet. Score 1, a few deposits in a small number of glomeruli; score 2, a few deposits in a large number of glomeruli; score 3, extensive deposits in a small number of glomeruli; and score 4, extensive deposits in a large number of glomeruli.

Hyalin cast. Score 1, seen rarely (less than ten tubules); score 2, seen in a small number of tubules; score 3, seen in many tubules; and score 4, seen in over half the tubules.

Dilatation and basophilic alteration and atrophy. Score 1, seen rarely; score 2, seen sometimes; and score 3, seen diffusely.

Cell infiltration (extensively monocytes, neutrophils and lymphocytes were rare). Score 1, scattered; and score 2, diffuse or crowded.

Fibrosis. Score 1, focal infiltration of fibroblasts, and score 2, diffuse infiltration of fibroblasts.

In all cases, score of 0 represents no changes.

Statistical analysis

Normally distributed data were expressed as mean ± SE. A one-way analysis of variance and Dunnett's test were used for multiple comparisons, and Student's *t*-test was used for the comparison of two groups where data were confirmed to be normally distributed. In the case of histological examination, differences among groups were compared using the non-parametric Mann-Whitney U test. A probability value less than 0.05 was regarded as significant.

Agents

CV-6504(HCl), CV-4151 [(E)-7-Phenyl-7-(3-pyridyl)-6-heptenoic acid], AA-861 [2-(12-Hydroxydodeca-5,10-dinyl)-3, 5, 6-trimethyl-1, 4-benzoquinone] and CV-3611 (2-O-Octadecylascorbic acid) were synthesized in the Chemistry Research Laboratories of Takeda Chemical Industries, Ltd. All agents except the kits from Wako and Amersham were purchased from Sigma Chemical Co. (St. Louis, Missouri, USA).

Results

Time course of the changes in various parameters in plasma, urine and renal cortex (Experiment 1)

The time course of the changes in various parameters in plasma, urine and renal cortical slices is shown in Table 1 and Figure 1. The BUN level was slightly but significantly increased day 1 after PAN injection, and the plasma cholesterol level was increased on day 3 (Table 1). However, the urinary excretion of protein was not significantly increased on day 1 or day 3. Massive proteinuria was observed on day 7 (Fig. 1 A and B) accompanied by marked increases in the level of plasma cholesterol, BUN (Table 1), plasma MDA (Fig. 1C) and plasma creatinine concentration and a decrease in the plasma albumin concentration (Table 1). The content of MDA in the renal cortex was not increased on day 1 or day 3, while it was significantly increased on day 7 (0.98 ± 0.05 nmol/mg protein; Fig. 1D). However, day 7 after PAN injection the production of MDA by incubated slices was not enhanced in the renal cortical slices from the PAN treated rats (0.63 ± 0.07 nmol/mg protein) in comparison with that in those from normal rats (0.90 ± 0.05 nmol/mg protein). Urinary excretion of MDA was increased on day 1 (9.4 ± 1.9 vs. 2.1 ± 0.4 nmol/100 g/24 hr in the normal rats), day 7 (16.4 ± 0.8 vs. 4.3 ± 1.4 nmol/100 g/24 hr in the normal rats) and day 14 (18.6 ± 1.1 vs. 4.2 ± 0.2 nmol/100 g/24 hr in the normal rats; Fig. 1C). In contrast, the production of TXB_2, 6-keto-$PGF_{1\alpha}$ and LTs induced by AA (20 μM) in the renal cortical slices was significantly increased starting day 1 and peaked on day 7 after PAN injection: day 1, 0.08 ± 0.03, 0.24 ± 0.06 and 16.9 ± 3.4 ng/mg protein for TXB_2, 6-keto-$PGF_{1\alpha}$ and LTs, respectively; day 3, 0.08 ± 0.01, 0.11 ± 0.02 and 21.2 ± 2.5 ng/mg protein for TXB_2, 6-keto-$PGF_{1\alpha}$ and LTs, respectively; day 7, 0.27 ± 0.07, 0.34 ± 0.08 and 23.7 ± 3.2 ng/mg protein for TXB_2, 6-keto-$PGF_{1\alpha}$ and LTs, respectively (Fig. 1 E and F). In the normal rats, these values were 0.01 to 0.04 ng/mg protein/30 min for TXB_2, 0.07 to 0.12 ng/mg protein/30 min for 6-keto-$PGF_{1\alpha}$ and 0 to 0.18 ng/mg protein/30 min for LTs. The basal values of TXA_2 and LTs in the absence of AA were slightly higher in PAN control rats (TXA_2; 0.006 ± 0.004 ng/mg prot/30 min, LTs; 0.015 ± 0.004 ng/mg prot/30 min; summation of values on 1, 3, 7 and 14 days after PAN injection, $N = 22$) than in normal rats (TXA_2; 0.002 ± 0.001 ng/mg prot/30

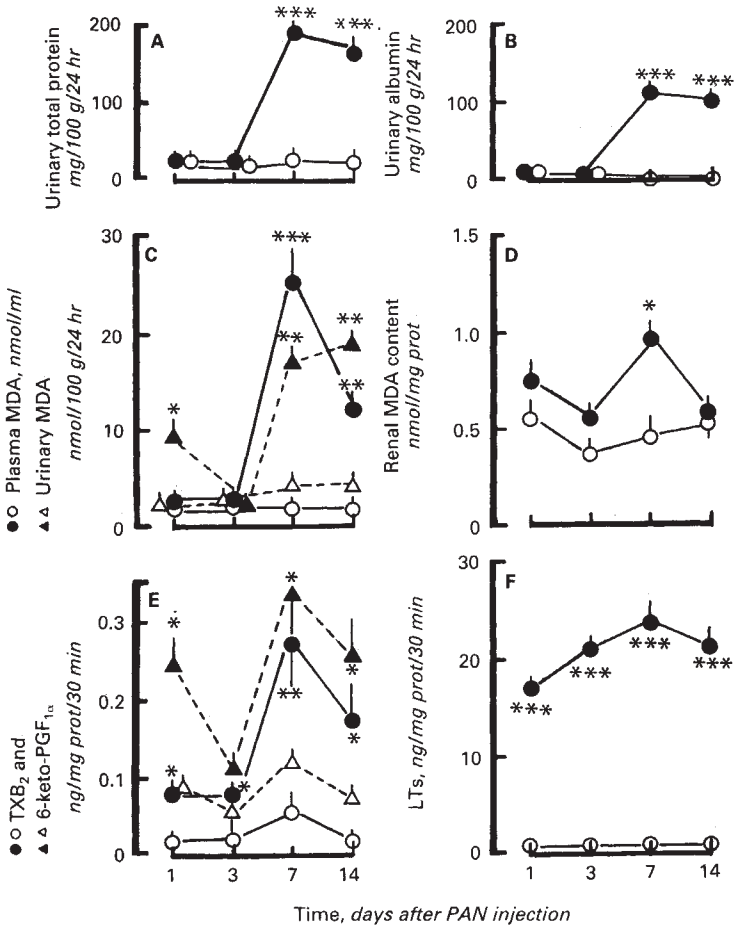

Fig. 1. *The time course of the changes in urinary protein, plasma MDA concentration, urinary MDA, renal MDA content and the production of renal TXA_2, 6-keto-$PGF_{1\alpha}$ and LTs in PAN treated rats (Experiment 1).* The closed circles and triangles show the values for PAN treated rats and the open circles and triangles show the values for normal rats. **(A)** Urinary total protein, **(B)** urinary albumin, **(C)** plasma MDA (●, ○) and urinary MDA (▲, △), **(D)** renal MDA content, **(E)** TXB_2 (●, ○) and 6-keto-$PGF_{1\alpha}$ (▲, △) produced from arachidonic acid in renal cortical slices, **(F)** LTs produced from arachidonic acid in renal cortical slices. The number of animals is 5 (day 1 – day 7) or 7 (day 14). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. normal rats (Student's *t*-test).

min, LTs; 0.006 ± 0.004 ng/mg prot/30 min, $N = 22$), but this difference was not significant.

Antiproteinuric action of CV-6504(HCl): Experiments 2 and 3

The quantity of urinary total protein and albumin in the normal rat was 16.6 ± 4.5 and 2.0 ± 0.7 mg/100 g/24 hr on day 7, respectively, and 20.3 ± 2.3 and 0.9 ± 0.3 mg/100 g/24 hr on day 14, respectively. In rats given PAN, urinary total protein and albumin excretion increased markedly, and reached a peak between days 7 and 14 after PAN injection (urinary excretion of total protein: 128.6 ± 11.3 and 103.6 ± 14.2 mg/100 g/24 hr on day 7 and day 14, respectively; urinary excretion of albumin: 77.6 ± 9.0 and 64.3 ± 10.2 mg/100 g/24 hr on days 7 and 14, respectively). CV-6504(HCl) at doses of more than 3 mg/kg (p.o.) suppressed the proteinuria. The effects at 10 and 20 mg/kg were remarkable (Fig. 2; urinary excretion of total protein: CV-6504(HCl) 3 mg/kg/day; 87.8 ± 13.5 and 47.5 ± 10.7, 10 mg/kg/day; 26.4 ± 4.1 and 28.5 ± 4.8, 20 mg/kg/day; 21.8 ± 5.1 and 26.3 ± 7.8 mg/100 g/24 hr on days 7 and 14, respectively; urinary excretion of albumin: CV-6504(HCl) 3 mg/kg/day; 52.6 ± 9.1 and 26.8 ± 7.3, 10 mg/kg/day; 10.5 ± 2.9 and 9.5 ± 2.9, 20 mg/kg/day; 3.4 ± 1.1 and 8.8 ± 5.9 mg/100 g/24 hr on days 7 and 14, respectively). CV-6504(HCl) did not affect urine volume (Table 2). In the case of Experiment 3 (the first administration of CV-6504(HCl) was 1 day after PAN injection), this drug also attenuated the proteinuria on day 7 and day 14 (albumin excretion: on day 7, 64.4 ± 10.0 for control; $N = 8$; and 5.9 ± 3.5 for CV-6504(HCl); $N = 8$; $P < 0.01$ vs. PAN-control; on day 14, 53.0 ± 8.2 for control; $N = 8$; and 5.1 ± 2.1 for CV-6504(HCl); $N = 8$; $P < 0.01$ vs. PAN-control).

Histological findings in the kidney: Experiment 2

The weight of the kidney tended to increase in the group receiving PAN alone: normal rats, 0.82 ± 0.08 g; PAN-control rats, 1.05 ± 0.28 g; CV-6504(HCl) (10 mg/kg/day)-treated rats, 0.96 ± 0.14 g (Table 2).

Light microscopy. On day 14 of the experiment, infiltration of inflammatory cells into the glomeruli was not observed, but, deposits of PAS-positive materials were observed in the glomeruli of the rats given PAN alone. The renal tubules also showed prominent changes such as hyalin cast formation, dilatation, basophilic alteration and atrophy. The infiltration of a few inflammatory cells into the interstitial cells and the proliferation of fibroblasts in the interstitial cells were observed in the control rats receiving PAN alone. The occurrence of hyalin casts and cell infiltration was significantly reduced in the CV-6504(HCl) treated group (Table 3).

Electron microscopy. Electron microscopic examination of the glomeruli in rats treated with PAN revealed a definite abnormality in the epithelial cells. The foot processes of the epithelial cells were replaced by an expanse of flattened epithelial cytoplasm in the PAN-treated rat (Fig. 3A). In addition,

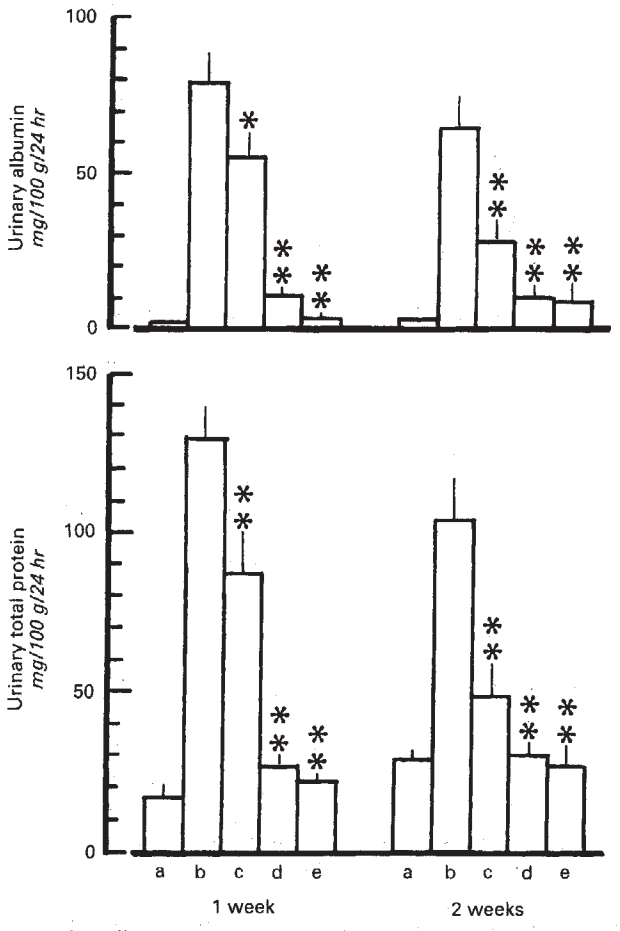

Fig. 2. *The effect of CV-6504(HCl) on puromycin aminonucleoside (PAN)-induced proteinuria in Experiment 2.* Abbreviations are: a, saline, $N = 10$; b, PAN, $N = 15$; c, CV-6504(HCl) 3 mg/kg, p.o., $N = 11$; d, CV-6504(HCl) 10 mg/kg, p.o., $N = 21$; e, CV-6504(HCl) 20 mg/kg, p.o., $N = 11$. All groups received a single intraperitoneal injection of PAN at a dose of 100 mg/kg body weight, except for the saline group which received a single intraperitoneal injection of saline. CV-6504(HCl) was administered 1 hour prior to PAN injection. The data shown are the means ± SE. $^*P < 0.05$, $^{**}P < 0.01$ vs. PAN control (Dunnett's test)

vacuoles and granular material were observed within the cytoplasm of the epithelial cell. The extent and severity of lesions in the epithelial cells in the rats treated with CV-6504(HCl) were significantly less than that in the control rat receiving PAN along (Fig. 3B). No endocapillary cell proliferation, mesangial abnormalities or sclerosis was observed in either group.

Body weight and plasma levels of albumin, cholesterol and BUN: Experiment 2

The body weight of PAN-treated rats was significantly lower than that of normal rats day 14 after PAN injection. CV-6504(HCl) inhibited this reduction in body weight (Table 2). The level of plasma albumin in the PAN group (3.11 ± 0.09 g/dl) was significantly lower than that in the normal rats (3.87 ± 0.09 g/dl), and the levels of plasma cholesterol (187.5 ± 21.4 mg/dl) and BUN (26.6 ± 2.2 mg/dl) in these rats were higher than those in normal rats (76.6 ± 4.5 mg/dl for cholesterol and 16.8 ± 1.5 mg/dl for BUN). Two week treatment with CV-6504(HCl) caused significant suppression of the plasma cholesterol and BUN levels (99.1 ± 10.1 mg/dl for cholesterol, $P < 0.01$, and 20.2 ± 0.6 mg/dl for BUN; $P < 0.05$; Table 2).

Levels of MDA, 11-dehydro-TXB_2 and LTs in plasma and urine: Experiment 2

As shown in Figure 4, the level of plasma MDA and urinary excretion of MDA were significantly higher in the PAN-treated control rats (12.5 ± 2.5 nmol/ml for plasma MDA and 19.0 ± 5.0 nmol/100 g/24 hr for urinary MDA) day 14 following PAN injection than in normal rats (1.0 ± 0.5 for plasma MDA and 4.2 ± 0.1 nmol/100 g/24 hr for urinary MDA). In the rats given CV-6504(HCl) (10 mg/kg, p.o.) for two weeks, these MDA levels were significantly lower (6.1 ± 1.5 nmol/ml for plasma MDA and 7.6 ± 0.5 nmol/100 g/24 hr for urinary MDA excretion) than those in the PAN-treated control rats. The levels of 11-dehydro-TXB_2 in plasma and urine tended to be higher in the PAN-treated control rats but not significantly so (plasma, 12 ± 3 for normal rats and 44 ± 18 pg/ml for PAN-control rats; urine, 598 ± 140 for normal and 799 ± 180 pg/100 g/24 hr for PAN-control rats), and the administration of CV-6504(HCl) for 14 days significantly decreased urinary 11-dehydro-TXB_2 (plasma, 25 ± 8 pg/ml, urine, 280 ± 80 pg/100 g/24 hr; $P < 0.05$ vs. PAN-control rats). The levels of LTs in plasma and urine were not higher in the PAN-treated control rats (plasma, 316 ± 110 for normal rats and 338 ± 55 pg/ml for PAN-control rats; urine, 1606 ± 238 for normal rats and 1594 ± 230 pg/100 g/24 hr for PAN-control rats).

Production of TXA_2 and LTs in renal cortical slices and MDA production and content in the renal cortical slices and tissue: Experiment 2

As shown in Figures 1 and 5, 14 days after the injection of PAN, the production of TXB_2 and LTs from AA in renal cortical slices was enhanced (0.17 ± 0.03 ng/mg/30 min for TXB_2 and 21.7 ± 2.6 ng/mg/30 min for LTs) as compared with that in the slices from normal rats (0.01 ± 0.01 ng/mg/30 min for TXB_2 and 0.13 ± 0.04 ng/mg/30 min for LTs). In the cortical slices from the rats treated with CV-6504(HCl) for two weeks, TXB_2 and LTs were not produced from AA (Fig. 5). The production of MDA in the renal cortical slices from the PAN-treated rats (0.24 ± 0.07 nmol/mg protein/45 min) was no higher than that in the slices from normal rats (0.32 ± 0.05 nmol/mg protein/45 min). The content of MDA in the renal cortex in the PAN-treated rats (0.58 ± 0.03 nmol/mg protein) was also no higher than that in normal rats (0.50 ± 0.06 nmol/mg protein) 14 days after PAN injection. The production of MDA in the slices and the content of MDA in renal cortex were not changed by the administration of CV-6504(HCl) (data not shown).

Inhibitory effect of CV-6504(HCl) on the production of TXA_2, LTs and MDA in the renal cortical slices or renal glomeruli in in vitro and ex vivo experiments (single administration): Experiment 4

One hour after CV-6504(HCl) (10 mg/kg, p.o.) was administered to the rats 14 days after PAN injection, the production of TXB_2 and LTs in renal cortical slices was inhibited by about 80% and about 60%, respectively (Fig. 6), and the production of both eicosanoids was also markedly inhibited in renal glomeruli

Table 2. Effect of two week CV-6504(HCl) administration on body weight, kidney weight, urine volume and the concentrations of plasma albumin, cholesterol, blood urea nitrogen (BUN) and creatinine in Experiment 2

	Body wt	Kidney wt	Urine volume	Plasma albumin	Plasma cholesterol	BUN	Plasma creatinine
	g		*ml/100 g /24 hr*	*g/dl*	*mg/dl*		
Normal (5)	218 ± 3	0.82 ± 0.08	7.2 ± 1.0	3.87 ± 0.09	76.6 ± 4.5	16.8 ± 1.5	0.398 ± 0.007
PAN rat (10)	173 ± 6	1.05 ± 0.28	6.7 ± 0.5	3.11 ± 0.09[a]	187.5 ± 21.4[b]	26.6 ± 2.2[a]	0.404 ± 0.012
PAN rat CV-6504 10 mg/kg (10)	198 ± 3[c]	0.96 ± 0.14	8.3 ± 0.9	3.77 ± 0.07[c]	99.1 ± 10.1[d]	20.2 ± 0.6[c]	0.401 ± 0.021

Abbreviation is: PAN rat, puromycin aminonucleoside (100 mg/kg, i.p.)-treated rats. The number of animals used is indicated in parentheses. Significance was by Student's *t*-test.
[a] $P < 0.05$, [b] $P < 0.01$ vs. normal rats
[c] $P < 0.05$, [d] $P < 0.01$ vs. PAN rats

(Table 4). The production of MDA was though not inhibited. However, when CV-6504(HCl) was added to the incubation medium of renal cortical slices from rats 14 days after PAN injection, it concentration-dependently inhibited the production of TXB_2 and LTs from exogenous AA with IC_{50} values of 4.8×10^{-8} M and 3.6×10^{-7} M, respectively, and also inhibited the production of MDA with an IC_{50} value of 5.0×10^{-7} M. CV-6504(HCl) did not inhibit the production of 6-keto-$PGF_{1\alpha}$ at concentrations of 10^{-6} to 10^{-4} M (data not shown).

Antiproteinuric effect of the combined administration of CV-4151, AA-861 and CV-3611: Experiment 5

In the rats 14 days after PAN injection, a single dose of CV-4151 (a TXA_2 synthetase inhibitor, 10 mg/kg, p.o.), AA-861 (a 5-lipoxygenase inhibitor, 300 mg/kg, p.o.) or CV-3611 (a radical scavenger, 50 mg/kg, p.o.) significantly inhibited the production of TXB_2, LTs and MDA, respectively, in renal cortical slices (Fig. 7) (Experiment 4). The inhibition of the production of TXA_2 and LTs caused by CV-4151 at 10 mg/kg and AA-861 at 300 mg/kg was almost equal to that caused by CV-6504(HCl). On the other hand, neither a single dose nor chronic administration of CV-3611 at 50 mg/kg (p.o.) reduced MDA levels in plasma or urine of PAN-treated rats (data not shown). Any of these three drugs alone slightly reduced the urinary protein. AA-861 or CV-3611 attenuated the proteinuria only in one of three experiments at 50 mg/kg (p.o.) and 300 mg/kg (p.o.) for 14 days, respectively (data not shown). However, the combined administration of two drugs (AA-861 + CV-4151 or AA-861 + CV-3611) did so slightly but significantly. The combination of all three drugs showed a clear antiproteinuric effect (Fig. 8).

Discussion

Although the levels of urinary and plasma 11-dehydro-TXB_2 (a stable metabolite of TXA_2 which is not affected by blood collection) and LTB_4 were not higher in PAN-control rats and the basal levels (without AA stimulation) of TXA_2 and LTs were not higher in renal cortical slices from PAN-control rats, the present study has revealed that MDA content and renal cortical production of TXA_2 and LTs from AA are enhanced in PAN-treated rats (Fig. 1). Since eicosanoids such as TXA_2 and

Table 3. Histological findings in the kidneys of rats with puromycin aminonucleoside-induced nephrosis: Effects of CV-6504(HCl) in Experiment 2

		The number of rats		
	Score[a]	Normal rats ($N = 5$)	PAN rats ($N = 10$)	CV-650 (HCl)-treated PAN rats ($N = 10$)
Glomerulus	4	0	5	4
PAS-positive	3	0	3	6
droplets	2	0	2	0
	1	0	0	0
	0	5	0	0
			c	c
Tubules	4	0	1	0
Hyalin cast	3	0	4	0
	2	0	4	4
	1	0	1	6
	0	5	0	0
			c	c,e
Dilatation	3	0	6	3
	2	0	3	4
	1	0	0	0
	0	5	1	3
			c	c
Basophilic	3	0	6	4
alteration	2	0	3	3
	1	0	1	3
	0	5	0	0
			c	c
Atrophy	3	0	2	0
	2	0	4	3
	1	0	2	6
	0	5	2	1
			b	b
Interstitial tissue				
Cell infiltration	2	0	7	2
	1	5	3	8
			b	d
Fibrosis	2	0	3	1
	1	0	2	1
	0	5	5	8

Five week old male Wistar rats were used. CV-6504(HCl) was administered 1 hr before puromycin aminonucleotide (PAN) injection and then administered daily for 13 days.
[a] The basis of the scores is described in **Methods**
[b] $P < 0.05$ and [c] $P < 0.01$ vs. normal rats; and [d] $P < 0.05$, [e] $P < 0.01$ vs. PAN rats (non-parametric Mann-Whitney U test)

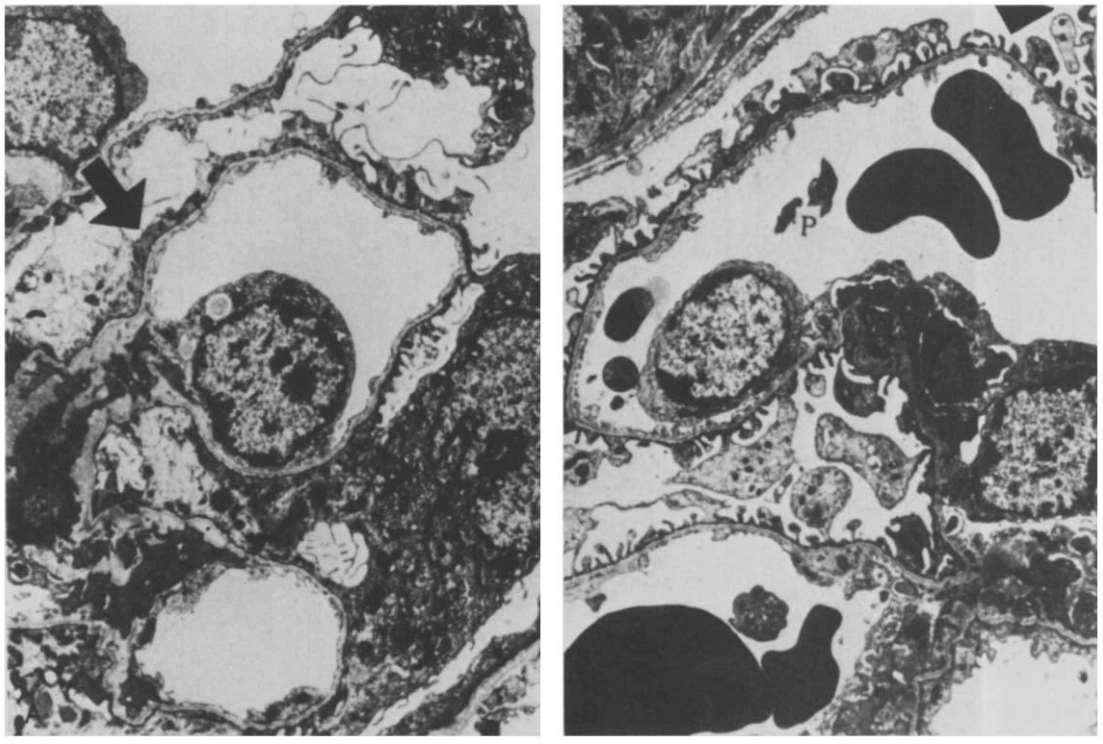

Fig. 3. *Electron microscopy of a kidney from a rat 14 days after the injection of PAN (Experiment-2).* **A.** PAN control rat. Fusion of foot processes is evident. **B.** CV-6504(HCl)-treated rat. Fusion of foot processes is much less evident. Arrows show the foot processes. P: platelet (× 7500).

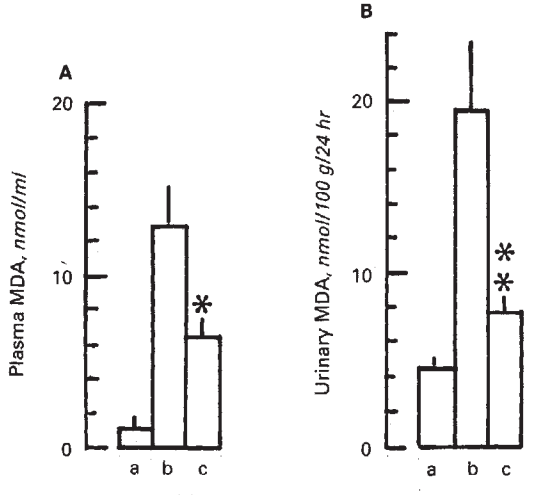

Fig. 4. *Plasma malondialdehyde (MDA) concentration and urinary MDA excretion day 14 after PAN injection (Experiment 2).* Abbreviations are: a, age-matched normal rats (N = 5); b, PAN control rats (N = 10; c, CV-6504(HCl) (10 mg/kg, p.o. for 2 weeks)-treated rats (N = 10); $^*P < 0.05$, $^{**}P < 0.01$ vs. PAN control rats (Dunnett's test)

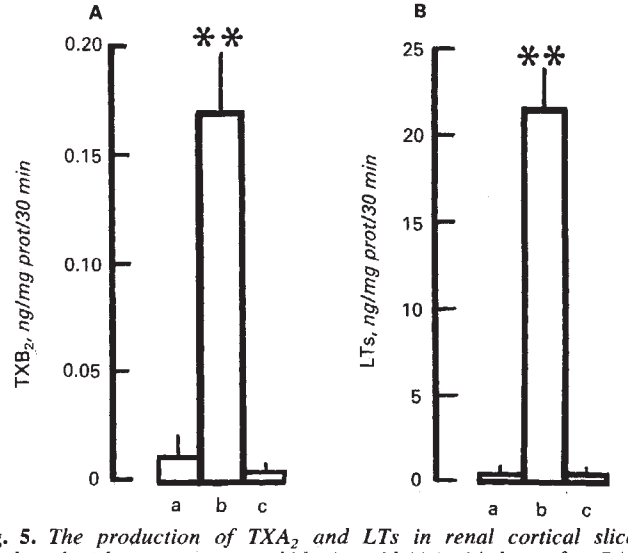

Fig. 5. *The production of TXA_2 and LTs in renal cortical slices incubated with exogenous arachidonic acid (AA) 14 days after PAN injection (Experiment 2).* Renal cortical slices were incubated with AA (final 20 μM) for 30 min at 37°C. TXB_2 and LTs in the incubation medium were measured by radioimmunoassay. Abbreviations are: a, normal rats (N = 5); b, PAN rats (N = 7); c, CV-6504(HCl) (10 mg/kg, p.o., for 2 weeks)-treated rats (N = 8); $^{**}P < 0.01$ vs. normal rats and CV-6504(HCl)-treated rats (Dunnett's test).

LTs are known not to be stored, it is reasonable that the basal level of these eicosanoids was not different between PAN-control rats and normal rats. The local production of these eicosanoids might be enhanced by some stimulation in PAN-control rats. The chronic administration of CV-6504(HCl), which inhibited the production of TXA_2 and LTs (in vitro and ex vivo) and MDA (in vitro), did remarkably attenuate the proteinuria. Even when CV-6504(HCl) was administered one day after PAN injection, it attenuated the PAN-induced proteinuria. Therefore, it is unlikely that CV-6504(HCl) affects the

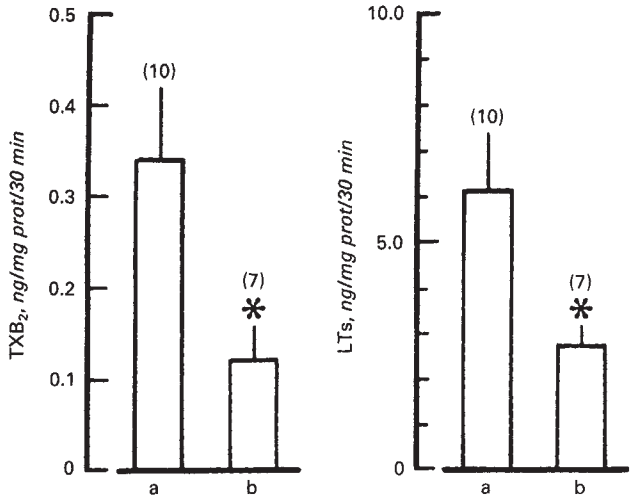

Fig. 6. *Inhibition of the production of TXA_2 and LTs in renal cortical slices following a single dose of CV-6504(HCl) given to rats day 14 after PAN injection (Experiment 4).* $^*P < 0.05$ vs. PAN control, CV-6504(HCl); 10 mg/kg, p.o. The kidneys were removed 1 hour after the administration of CV-6504(HCl). a, PAN-control; b, CV-6504(HCl).

Table 4. Inhibitory effects of CV-6504(HC1) on the production of thromboxane A_2 (TXA_2) and leukotrienes (LTs) in renal glomeruli of rats with puromycin-induced nephrosis in Experiment 4

	TXA_2 *pg/mg protein/30 min* AA-stimulated[a]	LTs *ng/mg protein/30 min* AA-stimulated
Normal (3)	9.4 ± 6.3[b]	0.04 ± 0.02[c]
PAN rats (8)	184.7 ± 55.7	22.40 ± 4.20
PAN rats with CV-6504(HC1) (8)	15.2 ± 8.9[b]	7.80 ± 2.90[b]

Basal level (without AA): TXA_2, 119.9 ± 32.9 pg/mg/30 min for PAN rats ($N = 16$), 18.9 ± 12.7 pg/mg/30 min for normal rats ($N = 3$). LTs were not detected in any group.

[a] AA; 20 μM arachidonic acid.

[b] $P < 0.05$, [c] $P < 0.01$ vs. PAN rats

delivery of PAN to the kidney or the uptake of PAN by the kidney.

Although it has been reported that oxygen metabolites [1–5], TXA_2 [6, 7] and LTs [8–11] may be potent mediators for the induction of proteinuria, there have been no reports demonstrating simultaneous enhanced renal production of TXA_2 and LTs in PAN-treated rats. Moreover, this is the first report showing the potent antiproteinuric effect of simultaneous inhibition of lipid peroxidation and the production of TXA_2 and LTs. We have, however, been so far unable to determine the precise mechanisms and the cell types responsible for the acceleration of the lipid peroxidation and the production of TXA_2 and LTs.

Since MDA is the final peroxidation product of phospholipids, an increase in the amount of MDA may indicate the enhancement of lipid peroxidation. Although the level of plasma MDA, urinary MDA excretion and MDA content in the renal cortex was increased by day 7 after PAN administration, the production of MDA by renal cortical slices was not enhanced in the PAN-treated rats. Furthermore, CV-3611 (a radical scavenger) did not attenuate the proteinuria and the increased MDA in plasma and urine, while in vivo administration of CV-3611 did reduce the production of MDA in renal cortical slices. On the other hand, chronic in vivo administration of CV-6504(HCl) did not inhibit the production of MDA in renal cortical slices, but the proteinuria was attenuated and MDA levels in plasma and urine were lower than in PAN-control rats. Therefore, it appears that the MDA detected in the plasma, urine and renal cortex is different from that found in the incubation medium of renal cortical slices. The increased MDA noted in urine of PAN-control rats may be a non-specific result of the presence of lipid-bearing proteins passively carried into the urine through a leaky glomerular basement membrane. Therefore, the reduction in the MDA concentration in urine by chronic administration of CV-6504(HCl) would be secondary to the reduction of the proteinuria. MDA in plasma may be derived from various tissues, including kidneys, heart and liver, damaged by PAN administration. Although the radical scavenging effect of CV-6504(HCl) in vivo or ex vivo was not observed in the present experiments, inhibition of the MDA production was observed when the drug was added in vitro to the incubation medium of renal cortical slices. Therefore, the radical scavenging effect might be partially involved in the antiproteinuric effect of CV-6504(HCl).

Although Thakur, Walker and Shah reported that the predominant radical in PAN nephrosis is the OH radical [5], it is unclear whether or not the generated MDA in plasma, urine and cortical slices is the OH radical and whether or not CV-3611 scavenges OH radicals.

The increase in renal MDA content might be due to an increased renal uptake of plasma lipoprotein, as the increase in renal MDA content was preceded by an increase in the plasma cholesterol level (Table 1, Fig. 1D).

As for LTs, Lefkowith, Morrison and Schreiner [8] reported that no synthesis of LTs from exogenous AA occurs in the glomeruli in normal rats. The amount of generated LTs in cortical slices from normal rats was about 1/100 of that in those from PAN-treated rats in the present experiments (Figs. 1F, 5). Although PAN-induced glomerular injury is known to be neutrophil-independent [37], there are extensive references to the rather severe interstitial infiltration in PAN nephrosis first described by Eddy and Michael [38], and to the glomerular accumulation of macrophages, described by Schreiner, Cotran and Unanue [37], and Diamond et al [39], all of which note 5- to 10-fold increases in the infiltration of leukocytes in both the glomerulus and interstitium and note increased production of lipoxygenase products, uniquely produced by leukocytes, and reactive oxygen species, also known to be produced by leukocytes. In the present experiments, infiltration of inflammatory cells was not observed in the glomeruli but was observed in the interstitial cells in this model on day 14 (Table 3). Therefore, these inflammatory cells may partially explain the cellular origin of LTs. On the other hand, since the accumulation of platelets was not observed, the resident renal cortical cells may be responsible for the production of TXA_2. It is uncertain whether the activities of 5-lipoxygenase and TXA_2 synthetase themselves are activated in the renal cortex of the PAN-treated rats. The increase in BUN on day 1 might be related to this increase in the production of TXA_2 and LTs, since TXA_2 and LTs are

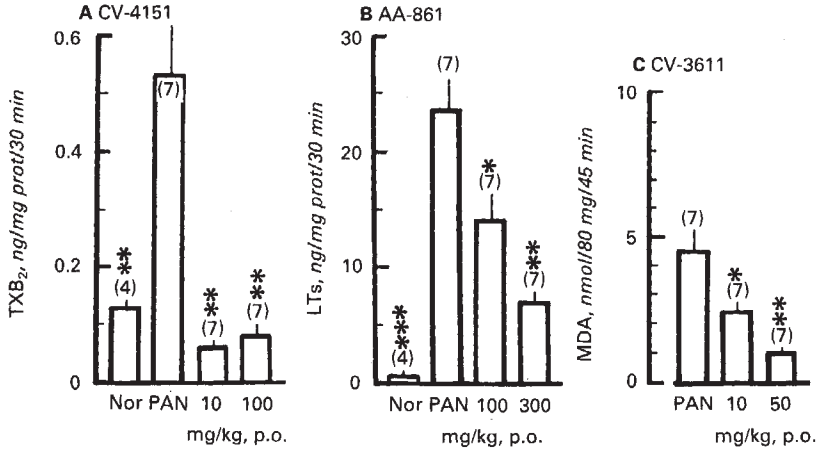

Fig. 7. *The inhibitory effect of CV-4151, AA-861 and CV-3611 on the production of TXA_2, LTs and MDA, respectively, in renal cortical slices from rats day 14 after PAN injection (Experiment 4).* $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. PAN control.

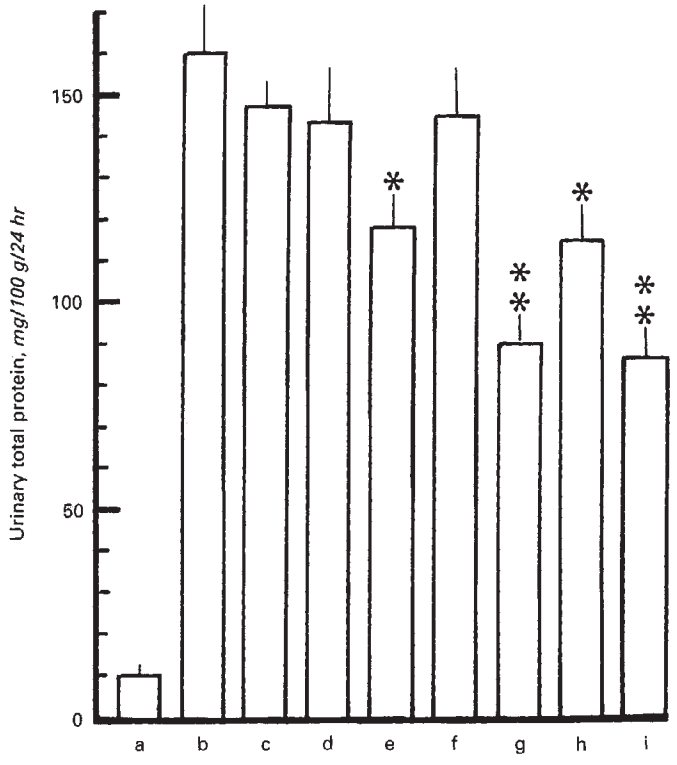

Fig. 8. *The effects of individual and combined administration of CV-4151, AA-861 and CV-3611 for 14 days after PAN injection on the proteinuria (Experiment-5).* Abbreviations are: (a) normal rats without PAN injection, (b) vehicle, (c) CV-4151 (10 mg/kg, p.o.), (d) CV-3611 (50 mg/kg, p.o.), (e) AA-861 (300 mg/kg p.o.), (f) CV-4151 + CV-3611, (g) AA-861 + CV-3611, (h) AA-861 + CV-4151 and (i) AA-861 + CV-4151 + CV-3611. The number of animals in each group was 7. $^*P < 0.05$, $^{**}P < 0.01$ vs. vehicle (Dunnett's test). Urinary excretion of total protein: (a) 11.0 ± 1.4, (b) 159.8 ± 11.8, (c) 145.6 ± 5.9, (d) 141.2 ± 13.2, (e) 116.2 ± 7.3, (f) 144.1 ± 11.8, (g) 89.7 ± 5.9, (h) 113.2 ± 8.8, (i) 85.3 ± 5.9 mg/100 g/24 hr.

known to cause a decrease in the glomerular filtration rate [6, 15, 16].

The antiproteinuric effect of CV-6504(HCl) was more potent than that of the combined administration of CV-4151, AA-861 and CV-3611. Therefore, the additional unknown action of CV-6504(HCl) may be attributed partially to the pathways allegedly affected by CV-4151, AA-861 and CV-3611.

In summary, the present study demonstrated that in rats with PAN-induced nephrosis, the ability of renal cortical slices and glomeruli to produce TXA_2 and LTs was enhanced, and MDA content was transiently increased. CV-6504(HCl) showed a very potent antiproteinuric effect, compared with a specific inhibitor alone or with the combination of two inhibitors, in rats with PAN-induced nephrosis. Although the detailed mechanisms of the interactions among TXA_2, LTs and free radicals are unclear at present, all three of these mediators may be involved in the initiation and the development of PAN-induced nephrosis.

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